In Vitro Interactions of Fusarium and Acanthamoeba with Drying Residues of Multipurpose Contact Lens Solutions

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PURPOSE. To examine in vitro effects of evaporation and drying of multipurpose contact lens solutions on survival of Fusarium and Acanthamoeba.

METHODS. Conidia of representative Fusarium from the 2004–2006 keratitis outbreak and trophozoites of Acanthamoeba castellanii were inoculated into commercially available multipurpose contact lens care solutions. These solutions were inoculated with 105–106 microbial propagules/mL and were evaporated for at least 24 hours. After drying, nutrient media for recovery of surviving organisms were added to the residues formed in the lids of 38 mm polystyrene Petri dishes. General morphologic patterns of the solution residuals and the distribution and morphologies of the microorganisms were recorded with microscopic imaging.

RESULTS. Various multipurpose contact lens disinfection solutions formed distinctive dried residual patterns. Both Fusarium and Acanthamoeba at concentrations tested above 104 per mL of disinfection solution were recovered from dried films with replicate testing. Mature cysts of Acanthamoeba not evident in the inocula were observed in sparse numbers in all dried solutions except one (Complete Moisture Plus; Advanced Medical Optics) and control salines where precysts and mature cysts were common. Both fusaria and amoeba tended to be observed in discrete regions of the dried residues.

CONCLUSIONS. Regions of drying films of multipurpose contact lens disinfection solutions on contact lens cases may induce and harbor dormant-resistant stages of Fusarium and Acanthamoeba. It is hypothesized that the evaporation and drying of multipurpose contact lens disinfection solutions may have been an added risk factor for case contamination among Fusarium and Acanthamoeba keratitis patients. The need for frequent replacement of contact lens cases is enforced. (Invest Ophtalmol Vis Sci. 2011;52:1793–1799) DOI:10.1167/iovs.10-5956

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survival of dormant and resistant cells; that is, chlamydoconidia of fusaria and cysts of Acanthamoeba (Zhang S, et al. IOVS 2008;49:ARVO E-Abstract 5521). This in vitro study examines drying film residues of several MPSs for potential roles in the development and sequestering of these dormant and resistant cells. We speculate that an evaporation scenario may be an added risk factor for rare contact lens-associated keratitis, particularly AK.

**METHODS**

Fusarium solani AFR4 and Fusarium oxysporum AFR9 from keratitis patients' cultures were obtained from the culture collection at Georgia State University. These cultures are representative of the F. solani–F. oxysporum species complexes isolated during the US 2004–2006 Fusarium keratitis outbreak. Acanthamoeba castellanii ATCC 30234 was obtained from the American Type Culture Collection. Conidia of the fusaria and trophozoites of the amoeba were grown, harvested, and suspended in phosphate-buffered saline (PBS; 8.0 g NaCl, 0.2 g KCl, 1.4 g NaHPO₄, 0.24 g KH₂PO₄ in 1000 mL distilled water, pH 7.4) as described previously. Conidia and trophozoites suspended in PBS were inoculated (10^4 μL) into 1.0–2.0 mL of various MPSs and a PBS control to give final cell densities ranging from 10^3 to 10^6 cells. MPSs examined included Complete Moisture Plus (CMP; Advanced Medical Optics, Santa Ana, CA), Optifree Express (OFE; Alcon Laboratories, Fort Worth, TX), Optifree RepleniSH (OFR; Alcon Laboratories), Aquify (AQ; Ciba Vision Corp., Duluth, GA), ReNu with MoistureLoc (RML; Bausch & Lomb, Rochester, NY), and ReNu Multiplus (RMP; Bausch & Lomb).

These solutions (approximately 1.5 mL) in lids of polystyrene Petri dishes (38 mm diam) or in wells (1.0 mL) of 12-well microtriter plates (Nalge Nunc Int., Tokyo), with and without the above inocula, were allowed to evaporate for at least 24 hours under a laminar-flow hood and in a chamber on a laboratory bench at ambient temperatures (22–24°C). The dried films overlaid with recovery broth were examined daily for at least 72 hours under the microscope for the emergence of trophozoites. Residue patterns in the lids and wells were examined progressively during the drying process, and patterns were recorded with a digital microscope system (KH-7700; Hirox-USA, River Edge, NJ). Multiple samples (n = 5–20) from at least five different containers of each MPS were examined. Noninoculated MPSs included samples from current and expired usage dates with replicate samples examined at different times. In all preliminary sampling, four major and consistent residual film patterns were discernable: CMP, RML/RMP, OFR/OFE, and AQ (Fig. 1). Representative sections of each dried MPS were studied further with wide-field microscopy, and selected areas were examined with scanning electron microscopy. The earliest time range for the microscopic observation of chlamydoconidia and mature cysts for inoculated films (including only MPSs within expiration dates) was recorded.

Fusaria were recovered from drying and dried MPSs with Sabouraud’s dextrose broth (SAB) and subsequent serial dilution and plating on Sabouraud’s agar. MPS solutions (1.0 mL) that had evaporated for 1, 2, and 4 hours in a laminar flow hood at 22–25°C were inoculated with approximately 10^4 microconidia and subjected to additional drying for 20 hours before addition of SAB. Trophozoites were recovered from the dried preparations by the addition of 1.0 mL of a peptone-yeast extract-glucose broth [PYG, proteose peptone 20 g, yeast extract 1.0 g, glucose 18 g in 900 mL distilled water (autoclaved)] supplemented with salts [0.98 g MgSO₄, 0.056 g CaCl₂, 0.01 g Na citrate. 0.34 g KH₂PO₄, 0.355 g Na₂HPO₄ in 100 mL distilled water (sterilized by filtration), or by the addition of 1.0 mL of sterile PYG without salts or 1.0 mL of sterile heat-killed cell suspension (~10^9 cells/mL) of Enterobacter aerogenes in water. In the preliminary experimental series conducted either in Petri dish lids or 12-well plates.

**FIGURE 1.** Typical MPS residuals and characteristic microscopy (×100) after 24 hours of evaporation at 22–24°C in Petri dish lids (approximately 1.5 mL in 38 mm diam lid). Residuals from five different lots of each MPS with different expiration dates (n = 10–20) were similar and distinctive.

**FIGURE 2.** Distinctive residual patterns of CMP inoculated with trophozoites after 24 hours of evaporation in 12-well plate wells (1.0 mL), showing alteration of residual pattern with higher inocula (A) 10^3 trophozoites and (B) 10^5 trophozoites (approximately ×5). Slower evaporation rates of CMP with increased inoculum density result in a less defined residual material structure compared with the finer dendritic formations seen in the lower inoculum level wells, suggesting that there is a higher retention of residual moisture. (C) Gross image of entire well showing 10^3 inoculum compared with (D) 10^5 inoculum.
no recoveries were noted with dried films with $10^2$ conidia or trophozoites, whereas inocula of $10^6$ gave consistent recoveries. Therefore inocula of $10^3$ and $10^5$ were routinely used in a test series with other densities used as indicated in the text.

**RESULTS**

Typical, representative dried film residues that formed within 24 hours in polystyrene Petri dish lids for each of the uninoculated MPSs are shown in Figure 1. All dried samples of MPSs, in both Petri dish lids and 12-well plates, produced readily discernable groups: CMP with staghorn-like crystalline structures, RMP/RML both with opaque but differently mottled films, and OFR/OFE and AQ demonstrating translucent films. These last three MPSs differed in the amount and appearance of liquid globules present in their films at 24 hours (Fig. 1). RMP and RML residues were separable also by their microscopic features (Fig. 1). Times for evaporation of 1.0 mL in the 12-well plates (25 mm diam) exceeded that for evaporation from the Petri dish lids at ambient temperatures by as much as 24 hours, particularly for the central inside wells and increasing inocula densities (Fig. 2). Additionally, determination of the distinctive microscopic features of the MPSs within the obvious visual groupings (RML/RMP, OFR/OFE and AQ) was more tedious with the 12-well plates because of their size and structural differences. The Petri dish lids were selected in this preliminary study for the more rapid screening of the MPSs.

**Fusaria**

Conidia tended to aggregate immediately after introduction into most MPSs, but some redistributed themselves into discrete areas around or in globules of the films during the drying process (Fig. 3). No viable fusaria at densities near the $10^4$–$10^5$ inoculation densities were recoverable after 24-hour exposures...
to nonevaporated MPSs in wells of 12-well plates or in MPS initial containers (Fig. 4). Additionally, no colony-forming units (CFUs) were obtained at these densities from dried MPS films that had been inoculated before the evaporation process. With partial evaporation of MPSs, before inoculation, low densities of CFUs from both species were obtained (Fig. 5). When microscopic observations and recovery procedures were extended over 48 hours and longer, CFUs were obtained only from F. oxysporum in dried RML and CMP. Conidia were observed in various areas, but the presence of chlamydoconidia was not confirmed with microscopy.

Amoeba

Trophozoites of A. castellanii, in part dependent on their density in the inoculum, tended to disperse, agglomerate, round up, or remain motile on inoculation into the various MPSs (Fig. 6). Trophozoites at densities near 10^4 or higher in PBS and CMP developed readily detectable mature cysts within 24 hours in all samples (n = 20) during the drying process (Fig. 7). The numbers of mature cysts that developed in the dried MPSs were classified as rare or common according to their ease of detection by microscopic observation. This was done because the clumping of cysts, the opacity of certain MPS films, and encapsulation of cysts within the dried MPS residues made quantitative evaluations difficult.

Mature double-walled cysts were most evident in PBS and CMP and sparsely observed in other MPSs (Fig. 8). While clumps of mature cysts were observed in dried PBS and particularly in CMP, single isolated cysts were eventually detected under the microscope in all “dried films” inoculated at densities of 10^6 to 10^9 cells but not at 10^2 and rarely at 10^3 densities. Recoveries of trophozoites of A. castellanii from dried films of selected MPSs at 24 hours in representative test series are shown in Table 1. The 24-hour evaporation of PBS and the MPSs in the Petri dish lids resulted in both lyses of trophozoites and relatively rapid induction of mature cysts.

The several recovery broths yielded similar data, except that the addition of heat-killed bacteria usually shortened the recovery time compared with PYG medium.

**DISCUSSION**

In aggregate, previously reported data have suggested that infectious keratitis in contact lens wearers occurs because microbes gain access to the contact lens paraphernalia, particularly the case during conditions of use and misuse of lenses. There the microbes can reproduce, attach to contact lenses stored in the case, and subsequently be transferred to the eye. In most cases, no clinically apparent infection ensues. In some cases, perhaps when host defenses are reduced because of systemic immunosuppression, local tear deficiency, or trauma to the epithelium, infectious keratitis develops. Species of Fusarium and Acanthamoeba, although common in the environment, are rarely the cause of contact lens–associated microbial keratitis. The rare involvement of these microorganisms may be attributed to their low virulence, common host-resistance factors, and possibly the susceptibility of most conidia, trophozoites, and precysts at low densities to most MPSs. We suggest, however, that the capacity of some strains to form dormant stages rapidly in drying MPSs increases their chances of survival in or on the contact lens case and the risk of potential involvement in disease. Our clinical isolates of Fusarium from the 2004–2006 keratitis outbreak have been observed to survive and amplify in partially dried MPSs after approximately 24 hours but not in nonevaporated MPSs. Levy et al. reported decreased efficacy of evaporated RML for a clinical isolate of Fusarium. Hume et al. reported that 2 of 10 clinical isolates of Fusarium were not susceptible to disinfection with current MPSs. About half of their 10 clinical isolates (with a modified inocula procedure that included hyphal elements with the conidia) did not meet the one log ISO standard disinfection criteria for most MPSs studied.
Our previous observations of ex vivo lenses indicated that the involvement of chlamydoconidia formation during conditions of actual use of MPSs cannot be excluded.11,17 Fusaria associated with the US 2004–2006 outbreak from varied geographical and clinical sources can attach to and penetrate hydrogel lenses and produce chlamydoconidia.12,17,46 We speculated that wearing of lenses penetrated by fusaria increased the risk of infection, but supportive data were sparse.17 Ultimately, the association of fusaria with worn contact lens may be most affected by personal hygiene practices and host-tear interactions.47

Our current data and the literature19,21,48 suggest that evaporation and drying of various MPSs induce Acanthamoeba trophozoites to form some dormant and mature (double-walled) cysts within 24 hours. Sriram et al.34 reported that mature or aged Acanthamoeba cysts in dried agar, including cysts formed by isolates from keratitis patients, remained viable after 20 years. As few as 100 mature cysts of Acanthamoeba have been shown to yield survivors after 6- and 24-hour exposures to the same MPSs we studied herein.48 Mature or double-walled cysts of wild-type Acanthamoeba are presumed highly resistant to adverse environmental conditions. We speculate that components of drying MPSs (e.g., propylene glycol derivatives complexed with hemicellulosomes in CMP) may harbor cysts and precysts of Acanthamoeba (and, in the case of the RML formulation the conidia or chlamydoconidia of fusaria) for extended periods in or on contact lens cases. Our data suggest that both rapid (within 24 hours) and delayed (~168 hours) induction and differentiation of trophozoites to mature cysts may occur within these drying residues. Possibly, excessive repeated rinsing with certain MPSs and air-drying of cases as currently recommended could, unexpectedly, build up residues that harbor dormant stages and increase the risk of contamination with microorganisms with lifecycle properties similar to those of Fusarium and Acanthamoeba. The irregular but common practice of “topping-off” (reuse of solution, a common practice identified among patients of both outbreaks) may also associate with the development of dried residues in and on the case.

Several investigations28,44 have reported that Acanthamoeba trophozoites form only immature cysts after short-term...

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* Number of dried films positive for trophozoite recovery/total number of films; densities of trophozoites developing from the positive recovery broths within 72 hours were highly variable; morphologies differed with the inocula and multipurpose solution.

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**FIGURE 8.** Mature (M) and immature (I) cysts of A. castellanii ATCC 30246 common in dried residues of CMP (A) and mature cysts of sparse occurrence in other MPSs such as OFR (B).

**FIGURE 9.** Cysts of A. castellanii observed in the mid-1980s (A) and 2010 (B) in dried residuals of peroxide products in contact lens cases of patients with Acanthamoeba keratitis. Dense numbers of cysts were present in both cases.
exposures to various MPSSs, particularly those containing PHMB. We observed, however, the formation of mature cysts, often in aggregates, in 24-hour dried residues of MPSSs, particularly CMP. This suggests that these areas of the “dried films” lacked or were deficient for their anti-amoeba properties. The water content of the films was in equilibrium with the ambient atmospheric conditions and hydroscopic components or regions of the “dried films” retained or adsorbed water throughout the study period. Certain “dried” residues retained or developed liquid globules with the rare presence of internal trophozoites. The extent to which our test conditions mimic in-use conditions for contact lens care with MPSSs is unknown.

Although undocumented, a decline in contact lens-associated AK in the United States was suggested in the early 2000s before the availability of CMP.5,4,7-9 The increasing reports of AK in 2006–2007 in the United States occurred in conjunction with decreased AIDS-associated infectious keratitis, increased “no-rub” MPSS use, the increased popularity of silicone hydrogel lenses, enhanced diagnoses and alertness to AK, and decreased quality of water in certain localities.5,13,15,21 The time span for both keratitis outbreaks overlapped (2004–2007), and epidemiologic evidence has shown each was associated with the use of a single but different MPSS with “topping-off” as a common risk factor.1,8 We relate “topping off,” in part, as a response to evaporation of the MPSS.

Trophozoites are considered the infective stage of Acanthamoeba with chronic and recurrent AK related, in part, to differentiation of cysts within corneal tissue.19,49 The infectious dose of trophozoites for contact lens-related AK in the United States is unknown, but AK may involve, in part, repeated exposure to high numbers of cysts and trophozoites from contact lens storage cases co-contaminated with repeated exposure to high numbers of cysts and trophozoites for contact lens-related AK in the United States is unknown, but AK may involve, in part, repeated exposure to high numbers of cysts and trophozoites from contact lens storage cases co-contaminated with bacteria and fungi.8,19,25,48 Several investigations have reported bacterial contamination of lens storage cases to be common (20%), with Acanthamoeba being recovered from 8% or more of the cases.51,50,51 Still, AK remains a rare disease, probably because of the common occurrence of antibodies for Acanthamoeba in the general population.19,21,52 The major upswings in AK in the United States among contact lens wearers were associated in the mid-1980s with the use of home-prepared and nonpreserved saline and in 2006–2007 with use of CMP.5,3,5,8,53 These outbreaks may both have been related more to sustained cysts of Acanthamoeba in contact lens cases than exposure to environmental sources of trophozoites or cysts (Fig. 9).

In summary, the characteristics of certain drying MPSS residues in and on contact lens cases may be significant risk factors for the development of microenvironments that sequester or harbor rare agents of infections. We present and review data that show survival of fusaria on and in cases with RML and the rapid development and aggregation of mature cysts in CMP, the dried residues of which often encapsulate the cysts. Such interactions of drying residues of certain MPSSs with some strains of Fusarium and Acanthamoeba suggest that some contact lens case cleaning and replacement recommendations be modified. The generally recommended practice for frequent replacement of lens cases is further supported.

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References


